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**14. ABSTRACT**
Poly(ADP-ribose) polymerase (PARP) inhibition provides a promising therapeutic modality for targeting homologous recombination (HR) deficient tumors such as BRCA1 and BRCA2-mutated triple negative breast cancers (TNBCs). Although PARP inhibitors have shown activity in the BRCA-associated TNBCs, several of these tumors develop de novo as well as acquired PARP inhibitor (PARPi) resistance. Besides attenuation in intracellular uptake of drugs, the only known mechanism that drives chemotherapy resistance of BRCA1/2-deficient cancers is through the restoration of HR. Recent studies from our laboratories (Nussenzweig and D'Andrea) indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render BRCA1/2-deficient cells resistant to PARPi in a HR-independent manner. The objective of our project is to collaboratively test the hypothesis that complex processes involving Alt-EJ or replication fork stability promote survival and drives resistance to chemotherapy. A detailed assessment of the critical mediators that regulate the balance between HR, Alt-EJ and replication fork degradation should identify novel means to overcome acquired chemoresistance in BRCA1/2-mutated breast cancers. During the first year of the DOD funding, we have made progress in identifying the proteins which contribute to the replication fork stability and we have identified new mechanisms of chemoresistance in BRCA2-deficient tumors.

**15. SUBJECT TERMS**
Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, EZH2, PAR, FK866, NMNAT-1/2/3

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## 1. INTRODUCTION:

The inactivation of the tumor suppressor genes *BRCA1* and *BRCA2* by mutations or epigenetic silencing is a critical event in breast and ovarian carcinogenesis. *BRCA1* and *BRCA2* encode proteins that are essential for accurate double strand break (DSB) repair by homologous recombination (HR). BRCA1 functions early during DSB resection, BRCA2 functions later in HR by catalyzing RAD51 nucleo-filaments at processed DSBs. Accordingly, HR deficient breast and ovarian tumors are highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors, since PARP inhibitors exhibit synthetic lethality in tumors with defective HR DNA repair. PARP inhibitors are currently in development for BRCA- or otherwise HR repair-deficient cancers, with FDA approval of Olaparib, Rucaparib and Niraparib. Of these drugs, Olaparib has been the most widely studied thus far, and it has been approved by the FDA as a monotherapy for treatment of ovarian cancer patients with germline *BRCA1* or *BRCA2* mutations. Nonetheless, *de novo* and acquired PARP inhibitor (PARPi) resistance, is a pressing clinical problem in patients with BRCA-deficient cancers treated with PARP inhibitors. Therefore, identification of the mechanisms underlying PARPi resistance is crucial for improving treatment and predicting tumor responses. Besides attenuation in intracellular uptake and increased efflux of drugs, the other known mechanism of PARPi resistance in BRCA-deficient tumors include restoration of HR due to somatic reversion of *BRCA1/BRCA2* or loss of other genes such as 53BP1, RIF1 or REV7. Recently, it was shown that BRCA1 and BRCA2 protect stalled replication forks from Mre11-mediated degradation, independent of their roles in HR. Accordingly, restoration of either HR capacity or replication fork stability is also associated with PARPi resistance in BRCA-deficient tumors. Indeed, a recent study from the Nussenzweig laboratory indicated that loss of *PTIP* protects replication forks from degradation in both *BRCA1*- and *BRCA2*- deficient cells and confers PARPi resistance. The D'Andrea laboratory has recently identified a novel DNA repair pathway, the so-called PARP/POLQ end-joining pathway, which, when upregulated, provides the HR-deficient breast tumor cell with an alternative mechanism of DNA repair. Collectively, recent studies from both laboratories indicate that deregulation of pathways that promote extensive degradation of nascent DNA strands and alternative end-joining (Alt-EJ) can render BRCA1/2-deficient cells resistant to PARPi in a HR independent manner. *We had therefore hypothesized that replication fork protection and PARP mediated Alt-EJ are novel and potentially interlinked mechanisms by which BRCA1/2-deficient breast cancers acquire resistance to chemotherapy.* Accordingly, the objective of our project is to provide a more detailed assessment of the factors that contribute to replication fork protection and Alt-EJ. This could lead to therapeutic approaches to overcome acquired resistance by targeting new vulnerabilities in both *BRCA1/2*-mutant and *BRCA1/2*–wildtype breast cancer.

## 2. KEYWORDS:

Breast cancer, BRCA1, BRCA2, PARP inhibitors, chemotherapy, resistance, HR, replication fork stability, EZH2, PAR, FK866, NMNAT-1/2/3

## 3. ACCOMPLISHMENTS:

The major goal of the project is to identify the molecular mechanisms of PARPi resistance in BRAC1/BRCA2 mutated breast tumors in order to improve therapeutic options for breast cancer patients.
The following specific aims were proposed:

> **Specific Aim 1**. Understand how PTIP-MLL3/4 and PARP1 confers chemoresistance and replication fork (RF) degradation in BRCA1/2-deficient cells.
> **Specific Aim 2**. Determine the interactions of BRCA2, FANCD2, and POLQ in replication fork (RF) stability and Alt-EJ
> **Specific Aim 3**. Assess mechanisms of PARPi resistance in mouse models and patient derived xenografts

- What was accomplished under these goals?

We have described major activities, specific objectives, significant results or key outcomes, conclusions and other achievements related to each specific aim in the following section. Both Drs. Nussenzweig and D'Andrea have noted tasks for which they were responsible (Site 1, NCI, NIH; Site 2, DFCI).

**Specific Aim 1**: Understand how PTIP-MLL3/4 and PARP1 confers chemo-resistance in BRCA1/2-deficient cells

**Major Task 1**. Defining the functional domains of PTIP and the contribution of MLL3/4 to drug resistance (**Site 1, NCI, NIH, Dr. Nussenzweig**)

We performed structure/function studies using PTIP mutants. To identify the region of PTIP that promotes RF degradation in *Brca2*-deficient cells, we expressed EV (empty vector), FL (full-length PTIP), W165R (disrupting interactions with PA1), W663R (disrupting interactions with 53BP1 at DSBs), or Del-BRCT5-6 (disrupting interaction with MLL3/4 independently of DSBs), in *Brca2/Ptip* doubly deficient cells. We observed that only reconstitution of *Brca2/Ptip*-deficient cells with PTIP-Del-BRCT5-6 maintained fork protection. This data was recently published (A. Ray Chaudhuri et al. Nussenzweig, Nature 2016).

Since the PTIP-Del-BRCT5-6 domain disrupts interaction with MLL3/4, we therefore tested whether the recruitment of MRE11 at stalled forks was dependent on MLL3/4. We observed that MRE11 association at RFs was dependent on MLL3/4 as monitored by iPOND and immunofluorescence analysis. We also observed an enrichment of H3K4me1 and H3K4me3 at nascent forks upon HU treatment that was PTIP- and MLL3/4-dependent. Thus, deposition of MRE11 on newly synthesized or stalled chromatin correlates with the establishment of H3K4me1 and H3K4me3 at RFs. This data was recently published (A. Ray Chaudhuri et al. Nussenzweig, Nature 2016).

To determine whether MLL4 contributes to degradation of stalled forks in *Brca*-deficient cells, we examined RF degradation in *Brca1*$^{-/-}$ *Mll4*$^{-/-}$ and *Brca2*$^{-/-}$*Mll4*$^{-/-}$ B cells. *Brca1*$^{-/-}$*Mll4*$^{-/-}$ and *Brca2*$^{-/-}$ *Mll4*$^{-/-}$ cells displayed a partial rescue of fork degradation To test whether MLL4 methyltransferase activity is critical, we targeted the catalytic SET domain of MLL4 in *Brca1*-deficient B cells. We observed a significant rescue of fork degradation in *Brca1*$^{-/-}$*Mll4*-SET$^{-/-}$ cells, suggesting that the methyltransferase activity is important for promoting fork degradation. In addition, we observed that *Brca2*$^{-/-}$ *Mll4*$^{-/-}$ B cells showed a partial rescue of chromosomal aberrations upon PARPi and cisplatin treatment compared with *Brca2*$^{-/-}$ cells alone. This data was recently published (A. Ray Chaudhuri et al. Nussenzweig, Nature 2016).

**Major Task 2**: Determine the mechanisms by which PARP1 modulates chemosensitivity in BRCA2 deficient cells

**Subtask 1**: To test whether PARP1 deficiency protects replication forks from degradation in the absence of BRCA2 (**Site 1, NCI, NIH, Dr. Nussenzweig**)

It is possible that PARP1 triggers genomic instability in Brca2 deficient cells because of excessive poly(ADP-ribose) synthesis or NAD+ depletion. It has been reported that levels of PAR are increased in Brca2 deficient cells, which may result in energy depletion. If such mechanisms reduce NAD+ and ATP levels in Brca2-deficient cells, it might be possible to promote fitness and/or reduce genome instability by overexpression with PARG (an enzyme which cleaves the linkage between ADP-ribose and acceptor proteins), or by supplementing cells with NAD (+) precursors nicotinamide riboside and nicotinamide mononucleotide.

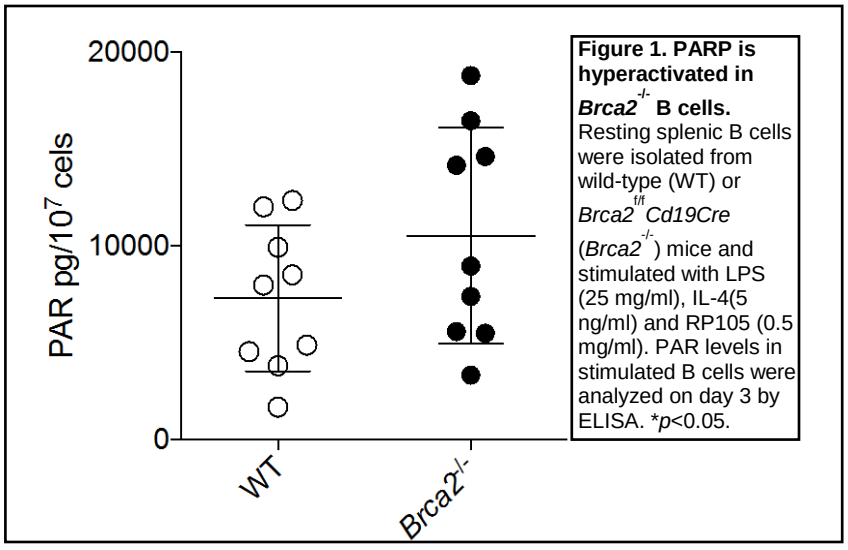


**Figure 1. PARP is hyperactivated in *Brca2*$^{-/-}$ B cells.** Resting splenic B cells were isolated from wild-type (WT) or *Brca2*$^{f/f}$*Cd19Cre* (*Brca2*$^{-/-}$) mice and stimulated with LPS (25 mg/ml), IL-4(5 ng/ml) and RP105 (0.5 mg/ml). PAR levels in stimulated B cells were analyzed on day 3 by ELISA. *$p$<0.05.

Poly ADP-ribosylation (PARylation) is a type of reversible posttranslational modification that is performed by enzymes in the PAR polymerase (PARP) family, which results in the covalent attachment of polymers of ADP-ribose units on a variety of amino acid residues on target proteins. This modification is mediated by a diverse group of ADP-ribosyl transferase enzymes that use ADP-ribose units derived from NAD+ to catalyze the ADP-ribosylation reaction. It has been previously estimated that ~90% of PAR polymers are formed via the catalysis of PARP-1, which is positively correlated with PARP activity. Activated PARP-1 and PARylation regulate various cellular machineries. Interestingly, it has been known that hyperactivation of PARP activity is observed in homologous

recombination (HR) defective cells, such as Brca2-deficient cells. Previous studies have also demonstrated that inhibiting PARP-1 activity or knocking out/silencing PARP-1 may rescue the genomic instability and viability of Brca2-deficient cells without restoring HR activity, suggesting that excessive PARylation may affect genomic instability observed in Brca2-deficient cells.

To confirm hyperactivated PARP activity in BRCA2 defective cells, we assessed PAR polymer in conditionally inactivated Brca2 B lymphocytes (Brca2f/fCd19Cre). We found increased levels of PAR proteins in Brca2-deficient cells reproducibly by ELISA (**Figure 1**).

PARPs are NAD+-dependent enzymes and thus require a source of NAD+ in all cellular compartments in which they function. Recent work has suggested that supplementation with NAD+ precursors nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) increase intracellular NAD+ levels in murine and human cells. In addition, it has been reported that FK866 effectively causes NAD+ depletion through specific inhibition of nicotinamide phosphoribosyltransferase (NAMPT), a key enzyme in the regulation of NAD+ biosynthesis from natural precursor nicotinamide. We, therefore, treated stimulated

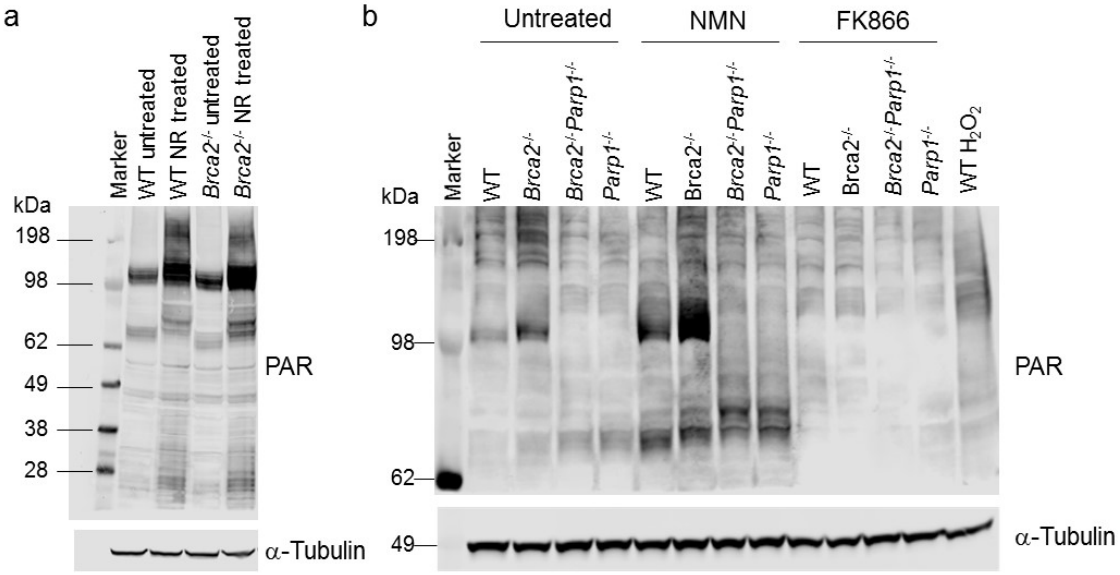


**Figure 2. NR, NMN or FK866 treatment modulates the levels of PARylation in stimulated B cells.** (a) Western blot analysis of PAR (top) and a-tubulin (bottom) in WT and *Brca2*$^{-/-}$ B cells. Cells were treated with NR (1 mM) for 16 h and collected on Day 3 after stimulation. (b) Western blot analysis of PAR (top) and a-tubulin (bottom) in WT, *Brca2*$^{-/-}$, *Brca2*$^{-/-}$*Parp1*$^{-/-}$, and *Parp1*$^{-/-}$ B cells. Cells were treated with NMN (500 mM) or FK866 (10 nM) for 16 h and collected on Day 3 after stimulation. As a positive control, WT B cells were treated with $H_2O_2$ (1 mM) for 10 minutes on day 3 after

B cells with NR, NMN or FK866 and analyzed the levels of PARylation. NR and NMN treatments increased the levels of PARylation, whereas FK866 treatment decreased the levels of PARylation in B cells (**Figure 2**).

These results indicate that cellular NAD+ levels maybe the limiting factor which can modulate the final PARylation output in the cells.

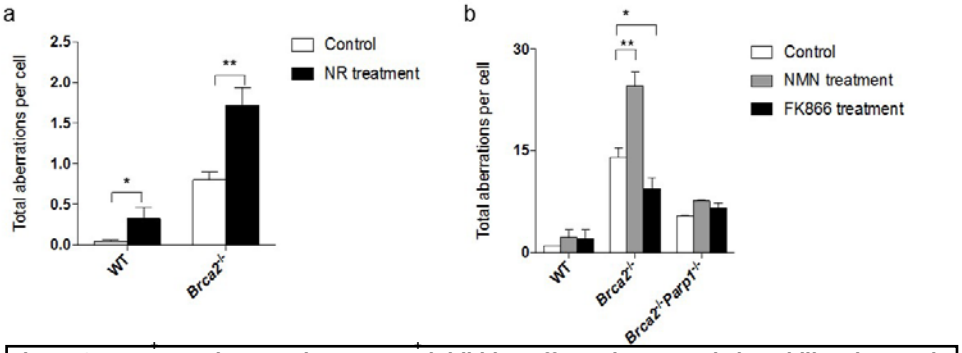


**Figure 3. NAD$^+$ supplementation or NAD$^+$ inhibition affects the genomic instability observed in *Brca2*$^{-/-}$ B cells upon cisplatin treatment.** (a) Genomic instability measured in metaphase spreads in WT and *Brca2*$^{-/-}$ B cells. LPS, IL-4 and RP105-stimulated B cells were treated with NR (1 mM) and cisplatin (0.5 mM) for 16 h and collected on Day 3. (b) Genomic instability measured in metaphase spreads in WT, *Brca2*$^{-/-}$, *Brca2*$^{-/-}$*Parp1*$^{-/-}$, and *Parp1*$^{-/-}$ B cells. Stimulated B cells were treated with NMN (500 mM) or FK866 (10 nM) with cisplatin (0.5 mM) for 16 h and collected on Day 3 after stimulation. *$p$<0.05, **$p$<0.01.

Next, we assessed chromosomal aberrations in cells treated with NR, NMN or FK866 in response to the DNA damaging agent cisplatin. NR and NMN treatment increased chromosomal aberrations in all genotypes and FK866 treatment decreased genome instability observed in Brca2-deficient cells (**Figure 3**). These results indicate that modulating cellular NAD+ levels through NAD+ supplementation increased cellular PARylation and enhanced genomic instability upon cisplatin treatment. In contrast, inhibition of NAD+ levels by FK866 treatment decreased the levels of PARylation and rescued genomic instability observed in Brca2-deficient cells.

Loss of Brca2 in embryonic stem cells (ESCs) is incompatible with cell survival. To test whether FK866 treatment could promote ESC survival, we used PL2F7 mESCs that have one functionally null allele of Brca2 - the other is a conditional knockout (cko) allele (Brca2cko/ko). We treated these cells with 10 nM of FK866 for 16 h and then deleted the cko allele by transient expression of CRE. After expression of CRE, we selected the recombinant clones in HAT (hypoxanthine, aminopterin and thymidine) media because CRE-mediated deletion of cko generates a functional HPRT minigene. Genotyping of the colonies did not reveal any Brca2ko/ko clones with DMSO treatment, consistent with the fact that BRCA2 is essential for viability. Remarkably, FK866 pretreatment results in a viable Brca2ko/ko cells in about 4% of clones in comparison to 0% of control (**Figure 4**), although rescued cells grew slower, and the colonies were smaller in size.

NMNAT-1, NMNAT-2, and NMNAT-3 comprise a small family of NAD+ synthases that produce NAD+ from NMN and ATP. NMNAT-1 is a central enzyme in NAD+ biosynthesis in the nucleus. To test whether NMNAT-1 deficiency in Brca2-deficient cells would have similar functional consequences as observed with NAD+ inhibitor, we knocked down Nmnat-1 in B cells using shRNAs. Absence of NMNAT-1 did not affect chromosomal aberrations observed in wild-type cells upon cisplatin treatment. In striking contrast, loss of NMNAT-1 in Brca2-deficient cells exhibited greater than 2- fold fewer chromosomal aberrations (**Figure 5**).

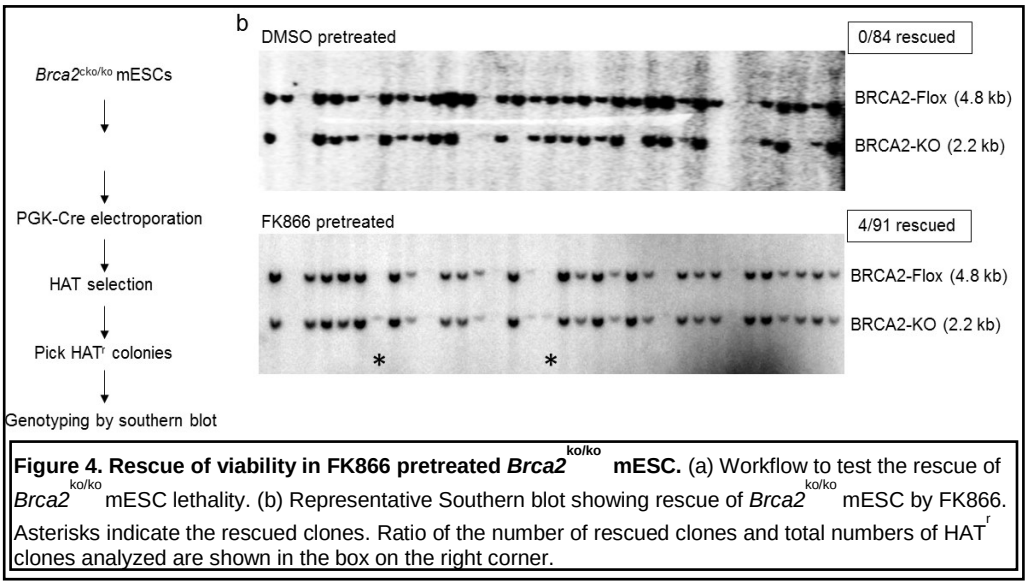


**Figure 4. Rescue of viability in FK866 pretreated *Brca2*$^{ko/ko}$ mESC.** (a) Workflow to test the rescue of *Brca2*$^{ko/ko}$ mESC lethality. (b) Representative Southern blot showing rescue of *Brca2*$^{ko/ko}$ mESC by FK866. Asterisks indicate the rescued clones. Ratio of the number of rescued clones and total numbers of HAT$^{r}$ clones analyzed are shown in the box on the right corner.

To analyze the impact of NMNAT-1 deficiency for the viability of Brca2-deficient ESCs, we generated three stably knockdown clones using three different shRNAs against Nmnat-1. We obtained several HAT-resistant mESC clones after cko deletion. Genotyping of the clones revealed that about 2% were Brca2ko/ko. Thus, deficiencies in NMNAT-1 rescue the lethality of Brca2-deficient ESCs (**Figure 6**).

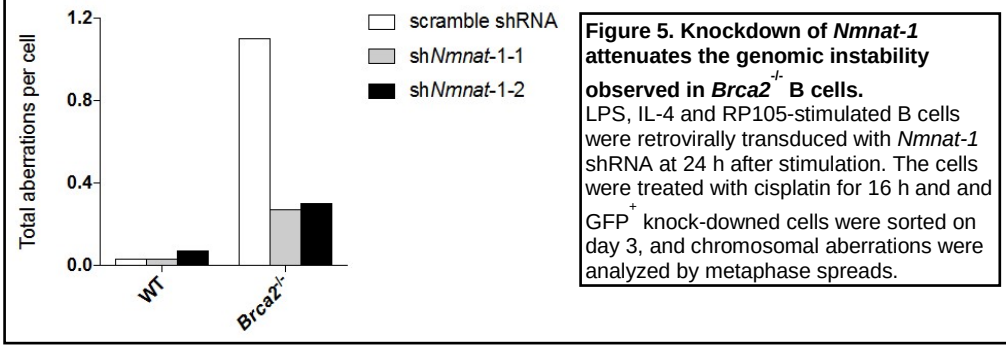


**Figure 5. Knockdown of *Nmnat-1* attenuates the genomic instability observed in *Brca2*$^{-/-}$ B cells.** LPS, IL-4 and RP105-stimulated B cells were retrovirally transduced with *Nmnat-1* shRNA at 24 h after stimulation. The cells were treated with cisplatin for 16 h and and GFP$^{+}$ knock-downed cells were sorted on day 3, and chromosomal aberrations were analyzed by metaphase spreads.

PAR polymers turn over rapidly in the cell. Thus, not surprisingly, a number of enzymes have evolved to remove covalently linked ADP-ribose and PAR from proteins. These PAR hydrolases include ADP-ribosyl hydrolase 3 (ARH3) and PAR glycohydrolase (PARG). Next, we wanted to down regulate cellular PAR levels by overexpressing PAR hydrolases in Brca2-deficient B cells and analyze genomic stability upon cisplatin treatment. We found that overexpression of PARG, but not ARH3, attenuates the chromosomal aberrations

observed in Brca2-deficient cells upon cisplatin treatment (**Figure 7**). These results again indicate that cellular PARylation levels affect genomic instability in Brca2-deficient cells.

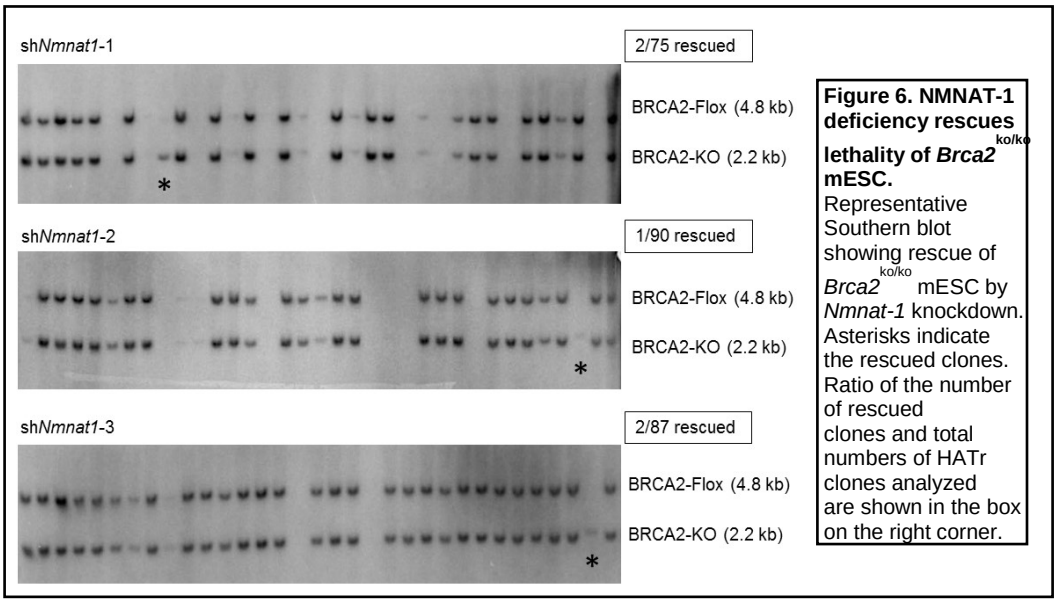


**Figure 6. NMNAT-1 deficiency rescues lethality of *Brca2*$^{ko/ko}$ mESC.** Representative Southern blot showing rescue of *Brca2*$^{ko/ko}$ mESC by *Nmnat-1* knockdown. Asterisks indicate the rescued clones. Ratio of the number of rescued clones and total numbers of HATr clones analyzed are shown in the box on the right corner.

**Milestone(s) Achieved**: These experiments have better defined the relationship between PARylation, Brca2-deficiency and genomic instability that lead to cancer development. Future studies will test whether NAD inhibition, or PARPG overexpression protects replication forks from degradation.

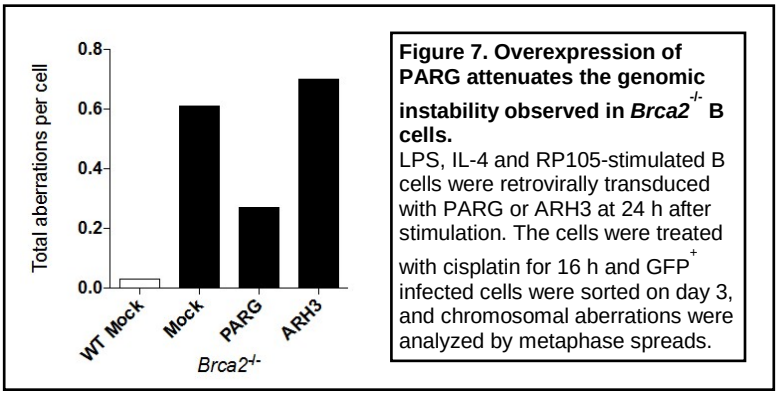


**Figure 7. Overexpression of PARG attenuates the genomic instability observed in *Brca2*$^{-/-}$ B cells.** LPS, IL-4 and RP105-stimulated B cells were retrovirally transduced with PARG or ARH3 at 24 h after stimulation. The cells were treated with cisplatin for 16 h and GFP$^{+}$ infected cells were sorted on day 3, and chromosomal aberrations were analyzed by metaphase spreads.

**Subtask 2a**: To submit and receive ACURO and HRPO approvals (proposed time line: 3-4 months) (**Site 2, DFCI, Dr. D'Andrea**)

**ACURO approval**: Importantly, we have already obtained an approval from the Institutional Animal Care and Use Committee (IACUC) (protocol # 08-036) for performing all of the proposed animal experiments at site 2, DFCI. The ACURO documents/forms have been submitted to DOD for approval. The USAMRMC Animal Care and Use Review Office (ACURO) has received the appropriate forms/documents involving animal studies and it is currently being reviewed. We have already addressed the comments after the initial review by the ACURO office and are currently waiting for approval. Animal work under the DOD funding will not be initiated until notification of approval from ACURO.

**HRPO approval**: We have already submitted Human Research Protection Office (HRPO) forms and approval has been obtained via phone conversation. It was determined that we need not submit any more documents for HRPO approval.

**Subtask 2b**: To generate PARP1/BRCA1 and PARP1/FANCD2 double knockout mice to monitor replication fork protection-250 mice (proposed time line: 12-18 months) (**Site 2, DFCI, Dr. D'Andrea**)

We are still waiting for ACURO approval. Therefore, these animal experiments have not been yet initiated.

**Milestone(s) Achieved**: ACURO forms for animal studies have been submitted and they are currently being reviewed. The HRPO approval has been obtained.

**Major Task 3**: Determine the changes in replisome composition associated replication fork degradation and protection

**Subtask 1**: Measuring MRE11 chromatin binding in BRCA2-, FANCD2-, BRCA2/PARP1-, BRCA2/PTIP-deficient cells treated with HU and cisplatin by high throughput microscopy (time line: 1-6 months). The progress is described in following section (Subtask 2) (**Site 2, DFCI, Dr. D'Andrea**).

**Subtask 2**: To determine the global composition of proteins at active and stalled replication forks using iPOND with mass spectrometry using WT vs. BRCA2- vs. FANCD2- vs. POLQ-deficient cells (proposed time line: 6-18 months) (**Site 2, DFCI, Dr. D'Andrea**).

Dr. D'Andrea has made significant progress related to this task. Indeed, we have validated known mechanisms of PARP inhibitor resistance (e.g. PTIP deficiency) using DNA fiber assays, biochemical assays and cell-based assays. In addition, we have identified a novel role of EZH2 in replication fork stability and have uncovered a new mechanism of PARPi resistance in BRCA2-deficient tumors.

We initially examined gene expression profiles of chromatin modifiers in cancers frequently associated with defects in HR (i.e. mutations in BRCA1/2 genes). EZH2 scored as the top overexpressed chromatin modifier in subgroups of ovarian, breast, and uterine cancers associated with HR deficiency. EZH2 expression correlated with tumor grade, POLQ and Ki67 expression (high POLQ expression is a surrogate marker for HR defect) and increase in tumors harboring alterations in BRCA1/2 genes. We therefore hypothesized that EZH2 may regulate genomic stability in BRCA1/2-deficient tumors. HR-deficient HeLa cells (siBRCA1 and siBRCA2) and HR-proficient control cells (siScr) were exposed to the EZH2 inhibitor GSK126, and clonogenic survival was measured under increasing concentrations of PARPi. While EZH2 inhibition had no effect on siScr or siBRCA1 transfected cells, it induced PARPi resistance in siBRCA2-transfected HeLa cells (data not shown). These results were recapitulated in a panel of BRCA2-deficient (e.g.VU423) but not BRCA1-deficient ovarian and breast cancer cell lines (**Figure 8, data not shown**). Interestingly,

EZH2 inhibition also conferred resistance of BRCA2-deficient cells to cisplatin (data not shown).

We next determined whether EZH2 depletion results in PARPi resistance in BRCA2-/- cells due to restoration of HR capacity or due to stablization/protection of replication fork. We performed a DR-GFP reporter assay and assessed RAD51 foci (a marker of HR activity) to evaluate HR status after EZH2 knockdown. EZH2 depletion did not increase HR frequency in BRCA1/2-deficient tumors nor it induced RAD51 foci after DNA damage indicating that PARPi resistance mediated by EZH2 loss does not result from restoration of HR (data not shown). By using the DNA fiber assay, we observed that EZH2 depletion or inhibition

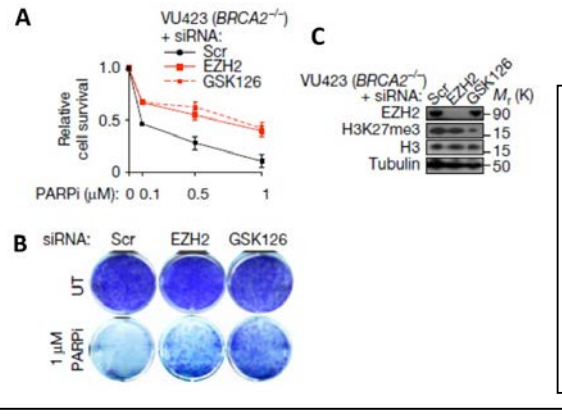


**Figure 8. EZH2 depletion or inhibition of EZH2 activity causes PARPi resistance if BRCA2-deficient breast cancer cells.** (A, B) Clonogenic assay and representative images of BRCA2-deficient VU423 cells expressing scrambled (Scr) siRNA, siRNAs against EZH2 or treated with GSK126 under increasing concentrations of a PARP inhibitor rucaparib. (C) Immunoblot analysis of cells expressing the above mentioned siRNAs at 72 hrs after transfection.

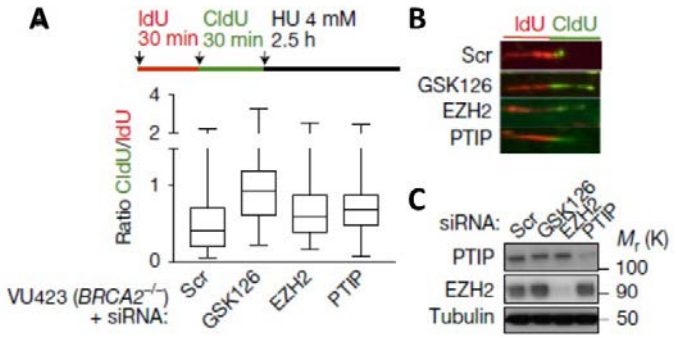


**Figure 9. EZH2 loss promotes replication fork protection in BRCA2-deficient tumors.** (A) Schematic and box plot for fork protection experiments. (B) Representative fibers (one over n=3 independent experiments) of BRCA2-deficient VU423 cells expressing scrambled (Scr) siRNA, siRNAs against EZH2 or PTIP or treated with GSK126 are shown. (C) Relative immunoblot analysis of the cells is shown.

increased replication fork protection after hydroxyurea (HU) treatment in BRCA2-deficient cells, similarly to PTIP knockdown (**Figure 9**).

Achievement of fork stability requires the simultaneous stalling of replication forks and the blockage of replication restart. Accordingly, EZH2 depletion or inhibition promoted fork stalling and reduced fork restart in BRCA2-deficient cells (data not shown). In addition, we determined that that EZH2 depletion increases fork protection and decreases fork restart specifically in BRCA2-deficient cells and independently of the MLL3/4-PTIP-MRE11 pathway. We next performed an accelerated native isolation of protein on nascent DNA (aniPOND) assay to

assess the localization of EZH2 and MUS81 at the replication fork. aniPOND is a biochemical assay that allows for the isolation and detection of proteins associated with sites of ongoing DNA replication. Using aniPOND experiments, we determined that EZH2 localizes to the stalled replication forks, recruits a nuclease MUS81 and promotes a degradation of stalled replication forks. Accordingly, low EZH2 levels reduce H3K27 methylation, prevent MUS81 recruitment at stalled forks and cause fork stabilization. As a consequence, loss of function of the EZH2/MUS81 axis promotes PARPi resistance in BRCA2-deficient cells. Using non-DOD funding, we have also confirmed these results in a murine model. We have determined that inhibition of Ezh2 in a murine Brca2-/- breast tumor model is associated with acquired PARP inhibitor resistance (data not shown, see Appendix).

Finally, to evaluate the impact of EZH2 loss *in vivo*, we analyzed the progression-free survival (PFS) after platinum-based chemotherapy (a surrogate measure of chemoresistance) of ovarian carcinoma patients harboring BRCA1/2 mutations. Interestingly, in BRCA2-deficient tumors, but not BRCA1-deficient or BRCA1/2-wild-type, low EZH2 expression correlated with poor response to chemotherapy (see Appendix for the data). These data suggested that low EZH2 levels worsen the prognosis of BRCA2-deficient patients by exhibiting chemotherapy resistance. Taken together, we identified EZH2 expression as a potential biomarker of BRCA2-deficient tumor response to chemotherapy.

**Milestone(s) Achieved**: We have identified a novel role of EZH2 at replisomes in BRCA2-deficient tumors. Specifically, we have determined that EZH2 localizes at stalled forks and mediates recruitment of the MUS81 nuclease. Low EZH2 levels prevent MUS81 recruitment at stalled forks and cause fork stabilization. As a consequence, loss of function of the EZH2/MUS81 axis promotes PARPi resistance in BRCA2-deficient cells. Accordingly, low EZH2 or MUS81 expression levels predict chemoresistance and poor outcome in patients with BRCA2-mutated tumors. We have identified EZH2 expression as a biomarker of BRCA2-deficient tumor response to chemotherapy.

**Specific Aim 2**: Determine the interactions of BRCA2, FANCD2, and POLQ in Replication Fork stability and Alt-EJ

**Major Task 4**: To determine whether the concurrent deletion of BRCA2 and POLQ results in synthetic lethality and reduced breast tumorigenesis

**Subtask 1**: To determine whether BRCA2 and POLQ are also synthetic lethal in tumorigenesis by using the conditional BRCA2/p53 knockout mouse model (K14CRE;BRCA2f/f;p53f/f, KB2P) (proposed time line: 12-15 months) (**Site 2, DFCI, Dr. D'Andrea**).

Conditional BRCA2/p53 knockout mice develop breast cancer within 100-300 days after birth. We aim to breed these mice with POLQ knockout mice and evaluate breast cancer progression in triple knockout mice. We anticipate that concurrent knockout of BRCA2/p53

and POLQ will result in synthetic lethality and the triple knockout mice will be tumor free. We are still waiting for ACURO approval. Therefore, these animal experiments have not been yet initiated. However, we have already sufficient number of POLQ knockout mice through our breeding colony of POLQ mutant mice. POLQ mice are being bred using a non-DOD funding source. Of note, during the first year of the DOD funding, we have confirmed all the cellular and biochemical phenotypes of POLQ deficiency.

**Milestone(s) Achieved**: We have confirmed a role of POLQ protein in Alt-EJ and in replication fork stability.

**Major Task 5**: To determine the mechanism of replication fork instability in FANCD2-deficient cells

**Subtask 1**: To test that PTIP/MRE11-mediated nucleolytic degradation is responsible for the replication fork instability in FANCD2-/- cells (proposed time line: 12-18 months). We had proposed to generate double knockouts in B-cells by crossing pTIPf/f-FANCD2-/- mice with the CD19 CRE transgenic mice and use B-cells from the double knockout mice for their competence in HR and fork stability. As we are still waiting for an ACRURO approval, these animal studies have not been initiated (**Site 2, DFCI, Dr. D'Andrea**).

**Milestone(s) Achieved**: None

**Major Task 6**: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ

**Subtask 1**: To identify and characterize proteins which cooperate with FANCD2 and POLQ in replication fork stability and Alt-EJ using iPOND/mass spectrometry (time line:6-18 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have not yet performed the experiments proposed for this subtask.

**Subtask 2**: Validation of FANCD2 and POLQ interacting proteins identified by mass spec using coimmunoprecipitation, shRNA knockdown, changes in POLQ nuclear foci and Alt-EJ template assays (proposed time line: 18-30 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have not yet performed the experiments proposed for this subtask.

**Milestone(s) Achieved**: None

**Specific Aim 3**: Assess mechanisms of PARPi resistance in mouse models and patient derived xenografts

**Major Task 7**: Evaluate replication fork stability relative to PARPi/cisplatin response in genetically engineered mouse models

**Subtask 1**: To test whether resistance in KB2P mouse models to PARPi correlates with RF protection and/or up-regulation of Alt-NHEJ using an organoid culture model to propagate tumors (proposed time line: 24-30 months) (**Site 2, DFCI, Dr. D'Andrea**).

We have set up immunohistochemistry (IHC)-based RAD51 foci assay to measure HR and a DNA fiber assay to measure RF protection in organoid cultures from primary ovarian cancer cells (see the details in "Major Task 8").

**Milestone(s) Achieved**: We have established assays in primary organoid cultures.

**<u>Major Task 8</u>**: Evaluate Replication Fork Stability and HR competence in PARP Inhibitor Sensitive and Resistant TNBC-PDX models

**Subtask 1**: To test a triple negative breast cancer-PDX model for resistance to PARPi and platinum by treating with vehicle, cisplatin, and olaparib and measuring RAD51 foci formation and replication fork fiber lengths (proposed time line: 30-36 months) (**Site 2, DFCI, Dr. D'Andrea**).

We aim to evaluate replication fork stability and HR competence in PARP inhibitor sensitive and resistant triple negative breast cancer (TNBC)-PDX models. We plan to test a triple negative breast cancer-PDX models for resistance to PARPi and platinum (Cisplatin) by treating PDX-bearing mice with vehicle, cisplatin, and olaparib and measuring RAD51 foci formation for HR competence and replication fork fiber lengths in tumors. Since we are still waiting for ACURO approval, these animal studies have not been initiated. Meanwhile, we have set up assays to evaluate replication fork stability and HR in tumor tissues. Importantly, we have developed a RAD51-based immunohistochemical (IHC) functional assay to determine HR-mediated DNA repair proficiency and PARP inhibitor sensitivity in PDX samples and primary tumors from ovarian cancer patients. Using this assay, we have determined that in ovarian cancer PDX models, presence of RAD51 foci correlates with PARP inhibitor response (see an example in **Figure 10**).

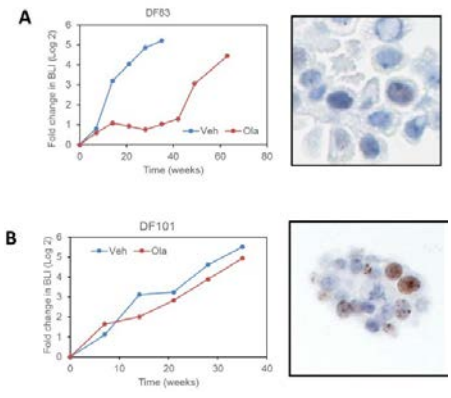


**Figure 10. Detection of RAD51 foci by immunohistochemistry in ovarian cancer PDX models.** (A) Olaparib sensitivity (left panel) and RAD51 staining of the DF83 PDX sample (right panel) are shown. Note the absence of RAD51 staining or RAD51 foci in this olaparib sensitive PDX model. (B) Olaparib sensitivity (left panel) and RAD51 staining of the DF101 PDX sample (right panel) are shown. Note the presence of RAD51 staining or RAD51 foci in this olaparib resistant PDX model.

We have also adopted the cell-based replication fork stability assay in primary organoid cultures derived from ovarian cancer patients and is currently optimizing it in PDX tumors.

**Milestone(s) Achieved**: We have established a RAD51 foci assay in primary PDX samples. In addition, we have established a replication fork stability assay in primary organoid cultures from ovarian cancer patients. Both of these assays will be useful in future in determining the replication fork stability and HR competence in PARP inhibitor or Cisplatin sensitive and resistant TNBC-PDX models

**Summary of the key research accomplishments**: During the first year of the DOD funding, we have made progress in identifying the proteins which contribute to the replication fork stability and we have identified a new mechanism of chemoresistance in BRCA2-deficient tumors. We have demonstrated that modulating cellular NAD+ levels can enhance or rescue genomic instability upon cisplatin treatment in Brca2-deficient cells. We have also determined that loss of function of EZH2 in BRCA2-deficient cancer cells promotes PARPi resistance. Importantly, our results identify EZH2 expression as a biomarker of BRCA2-deficient tumor response to chemotherapy. We have published a manuscript describing our EZH2 results.

- **What opportunities for training and professional development did the project provide**?

Nothing to report

- How were the results disseminated to communities of interest?

Results were shared with the scientific community via informal discussions, posters and presentations at scientific meetings and through publications in peer-reviewed journals

- What do you plan to do during the next reporting period to accomplish the goals and objectives?

The major goal of our project is to identify novel molecular mechanisms of PARPi resistance in BRCA1/2 mutated breast cancer. Therefore, during the upcoming grant funding year, we will rigorously study the interplay between PTIP, POLQ, PARP1, FANCD2, BRCA1/BRCA2 and EZH2/MUS81 axis in maintaining replication fork stability and Alt-EJ. In addition to the cell-based and biochemical assays such as iPOND and fiber assays, we will also use murine models for these studies. For example, we will generate PARP1/BRCA1 and PARP1/FANCD2 double knockout mice to monitor genomic stability and replication fork protection in the B-cells from these mice. We will also generate conditional triple knockout mice with deficiency for POLQ, BRCA2 and p53 in the breast tumors to determine whether POLQ and BRCA2 are synthetic lethal in tumorigenesis.

## 4. IMPACT:

Nothing to Report

**What was the impact on the development of the principal discipline(s) of the project?**

We have identified low EZH2 expression as a novel mechanism of chemotherapy resistance in BRCA2-deficient tumors. This finding has a high impact on identifying a patient population for a chemotherapy response. We have additionally demonstrated that modulating cellular NAD+ levels can enhance or rescue genomic instability upon cisplatin treatment in Brca2-deficient cells.

**What was the impact on other disciplines?**

Nothing to Report

**What was the impact on technology transfer?**

Nothing to Report

**What was the impact on society beyond science and technology?**

Nothing to Report

## 5. CHANGES/PROBLEMS:

**Changes in approach and reasons for change**

Nothing to Report

**Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report

**Changes that had a significant impact on expenditures**

Nothing to Report

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:**

Nothing to Report

**Significant changes in use or care of human subjects:**
Not applicable

**Significant changes in use or care of vertebrate animals:**

Nothing to Report

**Significant changes in use of biohazards and/or select agents:**

Nothing to Report

## 6. PRODUCTS

Publication: Journal publications.

Nothing to Report

**Books or other non-periodical, one-time publications**.

Nothing to Report

**Other publications, conference papers, and presentations**.

**Dr. Andre Nussenzweig (Site 1, NCI, NIH)**

1. Invited Speaker, 8th Biennial Workshop on the Clinical Translation of Epigenetics in Cancer Therapy, Atlanta, Georgia, USA, January 2017
2. Invited Speaker, Genome Dynamics in Neuroscience (GDN6), Hong Kong, China, January 2017
3. Invited Speaker, GRC-Physical Science of Cancer, Texas, USA, February 2017
4. Invited Speaker, Stem Cells & Cancer Gordon Research Conference, Lucca, Italy, February 2017
5. Invited Speaker, Keystone Symposia on Genomic Instability and DNA Repair, New Mexico, USA, April 2017
6. Invited Speaker, EMBO workshop on "Developmental Circuits in Aging", Athens, Greece, May 2017
7. Invited Speaker, DNA Replication as a Source of DNA Damage: From Molecules to Human Health, Rome, Italy, July 2017
8. Invited Speaker, CRUK/MRC Oxford Institute for Radiation Oncology, 4th Symposium, Oxford, UK, September 2017
9. Invited Speaker, EMBO Conference on ‘The DNA Damage Response in Cell Physiology and Disease’, Cape Sounio, Greece, October 2017
10. Invited Speaker, Meeting on the Biology of Aging, Groningen, Netherlands, October 2017

- **Website(s) or other Internet site(s)**

Nothing to Report

- **Technologies or techniques**

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Nothing to Report

- **Other Products**

Nothing to Report

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

| | |
|---|---|
| Name: | Andre Nussenzweig |
| Project Role: | Principal Investigator |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 3 |
| Contribution to Project: | Dr. Nussenzweig was responsible for the project management and collaboration with Dr. D'Andrea. |
| Funding Support: | Dr. Nussenzweig is supported by the NIH Intramural Research Program |

| | |
|---|---|
| Name: | Elsa Callen |
| Project Role: | Staff Scientist |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 3 |

| | |
|---|---|
| Contribution to Project: | Dr. Callen has been instrumental in developing the replication fork protection assay and in assessing fork stability in a variety of genetic contexts |
| Funding Support: | Dr. Callen is supported by the NIH Intramural Research Program |

| | |
|---|---|
| Name: | Kenta Shinoda |
| Project Role: | Post-doctoral fellow |
| Researcher Identifier (e.g. ORCID ID): | N/A |
| Nearest person month worked: | 6 |
| Contribution to Project: | Dr. Shinoda has performed work in the area of PARP1 biology and how affecting co-factor (NAD+) levels influences enzymatic activity and genome stability in BRCA-deficient cells |
| Funding Support: | Dr. Shinoda is supported by the NIH Intramural Research Program |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Dr. Arnab Ray Chaudhuri, a former post-doctoral fellow with Dr. Nussenzweig, is no longer associated with this project. Dr. Ray Chaudhuri has taken up an independent tenure-track position in the Department of Molecular Genetics at the University of Rotterdam in the Netherlands

**What other organizations were involved as partners?**

Nothing to report

## 8. SPECIAL REPORTING REQUIREMENTS:

**COLLABORATIVE AWARDS**: We have marked the tasks assigned to us and accordingly we have provided a progress made for each task.

**QUAD CHARTS**: Not applicable

**9. APPENDICES:** Nothing to report